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Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity.

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Peanut allergy is a significant health problem because of the prevelance and potential severity of the allergic reaction. Serum IgE from patients with documented peanut hypersensitivity reactions and overlapping peptides were used to identify the IgE-binding epitopes on the major peanut allergen, Ara h 1. At least twenty-three different linear IgE-binding epitopes, located throughout the length of the Ara h 1 protein, were identified. All of the epitopes were 6-10 amino acids in length, but there was no obvious sequence motif shared by all peptides. Four of the peptides appeared to be immunodominant IgE-binding epitopes in that they were recognized by serum from more than 80% of the patients tested and bound more IgE than any of the other Ara h 1 epitopes. Mutational analysis of the immunodominant epitopes revealed that single amino acid changes within these peptides had dramatic effects on IgE-binding characteristics. The identification and determination of the IgE-binding capabilities of core amino acids in epitopes on the Ara h 1 protein will make it possible to address the pathophysiologic and immunologic mechanisms regarding peanut hypersensitivity reactions specifically and food hypersensitivity in general.

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Recombinant peanut allergen Ara h I expression and IgE binding in patients with peanut hypersensitivity.

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Peanut allergy is a significant health problem because of the frequency, the potential severity, and the chronicity of the allergic sensitivity. Serum IgE from patients with documented peanut hypersensitivity reactions and a peanut cDNA expression library were used to identify clones that encode peanut allergens. One of the major peanut allergens, Ara h I, was selected from these clones using Ara h I specific oligonucleotides and polymerase chain reaction technology. The Ara h I clone identified a 2.3-kb mRNA species on a Northern blot containing peanut poly (A)+ RNA. DNA sequence analysis of the cloned inserts revealed that the Ara h I allergen has significant homology with the vicilin seed storage protein family found in most higher plants. The isolation of the Ara h I clones allowed the synthesis of this protein in E. coli cells and subsequent recognition of this recombinant protein in immunoblot analysis using serum IgE from patients with peanut hypersensitivity. With the production of the recombinant peanut protein it will now be possible to address the pathophysiologic and immunologic mechanisms regarding peanut hypersensitivity reactions specifically and food hypersensitivity in general

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REDUCTION IN IgE BINDING TO ALLERGEN VARIANTS GENERATED BY SITE-DIRECTED MUTAGENESIS: CONTRIBUTION OF DISULFIDE BONDS TO THE ANTIGENIC STRUCTURE OF THE MAJOR HOUSE DUST MITE ALLERGEN Der p 2

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(*First received* 5 *June* 1995; *accepted in revised form* 30 *October* 1995)

Abstract—Site-directed mutagenesis was used to investigate the contribution of disulfide bonds to the antigenic structure of Der p 2. Single amino acid variants were generated at cysteine residues, preventing the formation of disulfide bonds at positions 21–27, 73–78, and 8–119. The variants were tested for binding to murine monoclonal antibodies (mAb) and human IgE antibodies (Ab) in an inhibition enzyme immunoassay. Removal of the disulfide linking the amino–carboxy termini (C8–C119) had no effect on mAb binding, however, IgE Ab binding was reduced by up to 10-fold. The other two disulfides form small loops and disruption of these bonds gave different binding patterns. The variant lacking the C21–C27 bond showed up to a 40-fold reduction in antibody binding, while the variant lacking the C73–C78 bond showed more than a 100-fold reduction in IgE Ab binding and failed to bind 3 of 4 mAb. Intradermal skin testing with the C73–C78 variant supported the *in vitro* findings; the variant was 10 to 100-fold less reactive than rDer p 2. These two bonds thus make markedly different contributions to stabilizing the antigenic determinants of Der p 2. The results suggest that the C73–C78 bond plays a critical role in stabilizing the antigenic structure of this major mite allergen. Copyright © 1996 Elsevier Science Ltd.

Key words: IgE, antigenic structure, allergy.

INTRODUCTION

Inhalant allergens are an important group of antigens: approximately 15–25% of humans are naturally sensitized to common environmental antigens and produce specific IgE Ab and IgG Ab, and T cell responses to these proteins (Platts-Mills *et al.*, 1995; Rawle *et al.*, 1984; O'Brien *et al.*, 1992). The Ab response to allergens can now be studied in detail as many allergens have been purified to homogeneity, biochemically characterized, cloned and sequenced (Chua *et al.*, 1990a, 1990b; Yuuki *et al.*, 1991; Nishyama *et al.*, 1993). Our studies have focused on the 14 kD Group 2 allergens of the house dust mite, *Dermatophagoides pteronyssinus*, which are potent immunogens and elicit humoral and cellular responses in 80–90% of mite allergic individuals (Platts-Mills and Chapman, 1987; Lanzavecchia *et al.*, 1983; O'Hehir *et al.*, 1993; van Neerven *et al.*, 1993). Previous studies have shown that the B cell epitopes are heat and pH resistant, but are destroyed upon reduction and alkylation, suggesting that these determinants are conformational, and dependent on the tertiary structure of the protein (Lombardero *et al.*, 1990). This conclusion is supported by studies using polypeptide fragments produced from truncated Der p 2 cDNA that showed a low prevalence of IgE Ab binding (Chua *et al.*, 1991). Synthetic peptides spanning the entire Der p 2 sequence have also been used to map Ab binding regions, however, only one peptide, amino acids 65–78, retained IgE Ab binding, confirming that the majority of epitopes are conformational (van't Hof *et al.*, 1991). Taken together, these studies suggested that an alternative approach was required to investigate the conformational determinants on Group 2 allergens.

Site-directed mutagenesis has been used successfully for epitope mapping of antigens of known three dimensional structure (Smith *et al.*, 1991; Smith and Benjamin, 1991; Dudler *et al.*, 1994). The tertiary structure of Der p 2 is not known, however, this structure is stabilized by three disulfide bonds and the reduction and alkylation experiments suggested that these bonds are critical to the antigenic structure (Nishyama *et al.*, 1993; L mbardero *et al.*, 1990). We have systematically disrupted each f the three disulfide bonds (C8–C119, C21–C27 and C73–C78) to evaluate the contribution of each bond to the

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Abbreviations: mAb, m noclonal antibody (ies); Ab, antibody (ies); Ag, antigen; GST, glutathi ne-S-transferase; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electroph resis.

antigenic structure f the protein. Comparisons of the binding f human IgE Ab, *in vitro* and *in vivo*, as well as murine mAb binding, showed that each f the three disulfide bonds of Der p 2 is important to the antigenic structure, but the C73–C78 bond makes the major contribution to stabilizing the antigenic determinants of Der p 2. Furthermore, using site-directed mutagenesis it may bc possible to develop new approaches to allergen immunotherapy using variants that lack IgE Ab binding determinants.

MATERIALS AND METHODS

Oligonucleotide mutagenesis

The cDNA for Der p 2 was obtained from Dr Wayne Thomas, Perth, Australia, and subcloned into the bacteriophage M13mp19. Oligonucleotide directed mutagenesis was performed using the method of Kunkel and the Muta-gene Kit (Bio-Rad Laboratories, Richmond, CA), as previously described (Smith and Benjamin, 1991; Kunkel, 1985; Kunkel *et al.*, 1987). Oligonucleotides were synthesized at the University of Virginia Protein and Nucleic Acid Research Facility and were designed to substitute a variety of residues at positions 8, 21, 27, 73, 78 and 119, by providing a mixture of the four nucleotides (N) at the first and second positions of the target codon and dCTP, dGTP at the third position:

Cys8:	5′-C GAT GTC AAA GAT NNC/G GCC AAT CAT G-3′
Cys21:	5′-G GTA CCA GGA NNC/G CAT GGT TCA GAA CC-3′
Cys27:	5′-GGT TCA GAA CCA NNC/G ATC ATT CAT CGT GG-3′
Cys73:	5′-CCA AAT GCA NNC/G CAT TAC ATG AAA TGC-3′
Cys78:	5′-GC CAT TAC ATG AAA NNC/G CCA TTG GTT AAA GG-3′
Cys119:	5′-GGT GTT TTG GCC NNC/G GCT ATT GCT ACT CAT GC-3′

Mutants were identified by DNA sequence analysis, amplified with specific N and C terminal primers in a standard PCR reaction, and subcloned into pGEX2T (Pharmacia Biotech Inc., Piscataway, NJ).

Production and purification of recombinant (r) Der p 2

The plasmid pGEX2TP2 containing the native or mutated sequences, was introduced into the *E. coli* strain TG1, and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to logarithmic phase cultures. Briefly, an overnight culture was diluted 1:50 into Luria Broth and grown at 37°C with shaking until the OD_{600}nm of the culture reached 1–2. IPTG was added to a final concentration of 1 mM and cultures were continued 4–6 hr. Bacterial cell pellets from 1 l cultures were resuspended in 10 ml PBS (0.01 M Phosphate Buffer, pH7.4, 0.15 M NaCl) and lysed by the addition of lysozyme (1 mg/ml), fr zen at −20°C, and then thawed at room temperature. Fifty units of deoxyrib nuclease I (DNase I, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and the slurry was incubated at room temperature for 15 min then centrifuged at 12,000*g* for 15 min. The supernatant was passed over a glutathione–agarose c lumn (Sigma, St Louis, MO) equilibrated in PBS. The column was washed with five volumes of PBS-1% Triton X100, followed by two column volumes of PBS, and eluted with 10 mM glutathione (reduced form) in 50 mM Tris–HCl, pH7.5. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) and purity was assessed by SDS–PAGE on a PhastSystem (Pharmacia Biotech, Piscataway, NJ) and stained with Coomassie Brilliant Blue. Purified proteins were stored at −20°C. The unmodified glutathione-S-transferase (GST) fusion protein used for Ab binding studies was designated rDer p 2; variants were designated by the single letter amino acid code for cysteine (C), the position in the primary amino acid sequence of that cysteine, followed by the variant amino acid in single letter code, e.g. C21S is the serine (S) substitution at position 21.

Murine and human Ab to mite allergens

The specificity of the murine mAb used in this study has been described previously (Heymann *et al.*, 1989; Ovsyannikova *et al.*, 1994; Akagawa *et al.*, 1991). The mAb αDpX (provided by Dr Rob Aalberse, Amsterdam The Netherlands) and 7A1 define non-overlapping epitopes on Der p 2. The mAb 15E11 and 13A4, which define overlapping epitopes with αDpX and 7A1, respectively, were raised against Der f 2 and kindly provided by Dr Hirokazu Okudaira, Tokyo, Japan. The antibodies were used as 50% ammonium sulfate fractions of ascites. Sera from 40 patients with asthma and 40 patients with atopic dermatitis were selected as a source of IgE Ab. Collection of sera used in this study was approved by the Human Investigation Committee of the University of Virginia. Patients were skin test positive to *D. pteronyssinus*, and/or RAST positive to Group 2 allergens (Duff *et al.*, 1993; Gelber *et al.*, 1993; Sporik *et al.*, 1990). None of the patients were receiving immunotherapy at the time the serum was collected.

Monoclonal antibody modified RAST assay

The anti-Der p 2 mAb 7A1 was coupled to cyanogen bromide activated filter paper discs. After drying, the discs were incubated for 4 hr at room temperature with 100 μl *D. pteronyssinus* extract diluted to contain 1 μg/ml Der p 2 washed, and incubated with human serum diluted 1/10 and 1/50 in horse serum. After incubation for 18 hr at room temperature, discs were washed and incubated with ^{125}I-goat anti-human IgE (10^5 cpm/100 μl) for 6 hr. Discs were washed, transferred to assay tubes and counted. A standard curve was established with a serum pool from mite allergic patients that had been previously assayed against the WHO/IUIS *D. pteronyssinus* reference serum (NIBSC82/528) and was assigned 1000 units/ml IgE Ab to Der p 2 (Pollart *et al.*, 1988).

Direct binding enzyme immunoassay

The reactivity of rDer p 2 with mAb and IgE Ab (serum pool described above) was evaluated in a direct binding ELISA. Antigen was applied t a microtiter plate using 50 μl/well of a 20 μg/ml solution in PBS. After blocking non-reacted sites with PBS containing 0.05 Tween20 and 1% BSA (PBS–Tween, 1% BSA), the mAb or serum pool were added using serial two-fold dilutions. Ab binding was detected using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE Ab, along with 1 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS, Sigma A-1888) in 70 mM citrate-phosphate buffer, pH4.2 containing 0.03% H_2O_2 (Ovsyannikova *et al.* 1994). The results were evaluated at OD_{405}nm using a Titertek Multiscan Plus plate reader.

Inhibition enzyme immunoassay

The relative ability of variant antigen to interact with mAb and IgE Ab was determined by competitive inhibition in a modified ELISA assay as follows: antigen was mixed with Ab to give a final Ag concentration of 0.01–100 μg/ml. The Ab concentration was predetermined to give an endpoint OD_{405} of 1 in the ELISA in the absence of any inhibitor. All Ag and Ab solutions were prepared in PBS–Tween, 1% BSA. The Ag–Ab mixes were allowed to equilibrate overnight at 4°C and then were pipetted, in duplicate, into wells of a plastic microtiter plate that had been coated with rDer p 2 at 20 μg/ml. The plates were incubated at room temperature for 2 hr and then processed as a standard ELISA (described above) using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE as the secondary antibody. The positive control was rDer p 2 as inhibitor and the negative controls were GST and PBS-Tween, 1% BSA. Results were expressed as the percent inhibition of the reaction of Ab in the absence of any inhibitor.

Quantitative intradermal skin testing

Skin testing of patients using recombinant allergens was approved by the University of Virginia Human Investigation Committee (protocol 6440). rDer p 2 and C78G were prepared as described above, filtered over a 0.2 μm filter, and diluted to 5 μg/ml in sterile 0.4% phenol/1% human serum albumin saline. Subjects were skin prick tested with these solutions and then injected intradermally with 0.03 ml of dilutions of rDer p 2 and C78G, starting at 10^{-4} μg/ml and increasing the concentration (10-fold dilutions) until the reaction wheal reached 8 × 8 mm.

RESULTS

Reactivity of human IgE Ab and murine mAb with rDer p 2

A monoclonal antibody m dified RAST was used t c mpare the binding of IgE Ab to the native and rec m-binant Der p 2 by 80 sera fr m patients with asthma or atopic dermatitis. Figure 1 shows a strong quantitative correlation between IgE binding to Der p 2 and rDer p 2 ($r^2 = 0.77$, $p < 0.001$). In a direct binding ELISA, IgE Ab in a serum pool from seven patients gave overlapping binding curves to Der p 2 and rDer p 2 (Fig. 2A). The mAb αDpX, 7A1, 15E11, and 13A4 gave parallel binding curves using rDer p 2 (Fig. 2B).

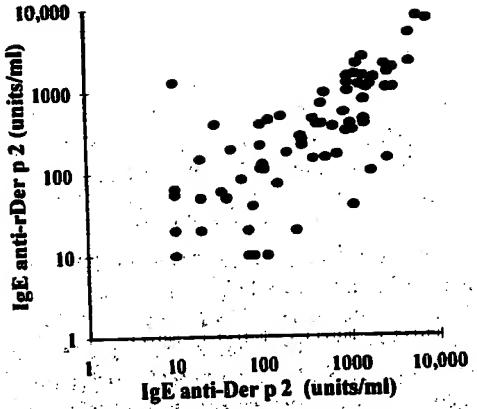

Fig. 1. Correlation between IgE Ab binding to native and recombinant Der p 2. A mAb modified RAST was used to measure IgE Ab binding to Der p 2 and rDer p 2 in sera from 80 patients with asthma or atopic dermatitis. Linear regression analysis showed an excellent correlation between IgE Ab binding to the two proteins ($r = 0.77$, $p < 0.001$).

Generation of rDer p 2 variants

Oligonucleotides were designed to give all 19 amino acid substitutions at each target cysteine and selected variants are shown in Table 1. Bradford Assay and SDS–PAGE analysis of the variants indicated that they were produced in approximately equal amounts from bacterial expression cultures, 1–4 mg/l after affinity purification (Fig. 3).

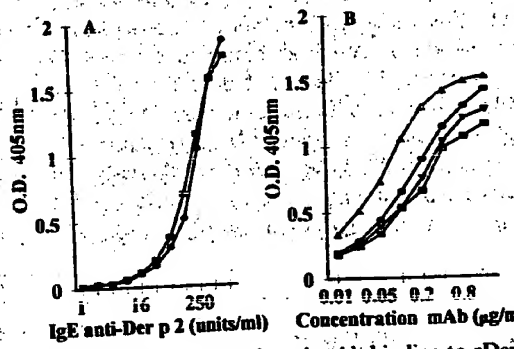

Fig. 2. Comparison of IgE Ab and mAb binding to rDer p 2. Binding curves for IgE Ab and murine mAb were compared by ELISA using plates coated with rDer p 2. Figure 2A compares IgE Ab fr m a serum pool of seven *D. pteronyssinus* allergic patients binding to Der p 2 ( ) and rDer p 2 (■). Figure 2B compares reactivity of 4 mAb with rDer p 2: mAb αDpX (●). mAb 7A1 (▲), mAb 13A4 (▼) and mAb 15E11 (■)

Table 1. Recombinant Der p 2 variants at cysteine residues generated by olig nucleotide-directed mutagenesis

Cysteine Residue[a]	Variant
8	**Gly**[b]
21	**Ser**
21	Arg
21	Leu
27	**Gly**
27	Trp
73	Arg
73	Leu
78	**Gly**
119	**Tyr**
119	Pro

[a]Disulfide bonds join cysteine residues 8–119, 21–27 and 73–78 in Group 2 proteins.
[b]Variants shown in bold were evaluated by inhibition ELISA.

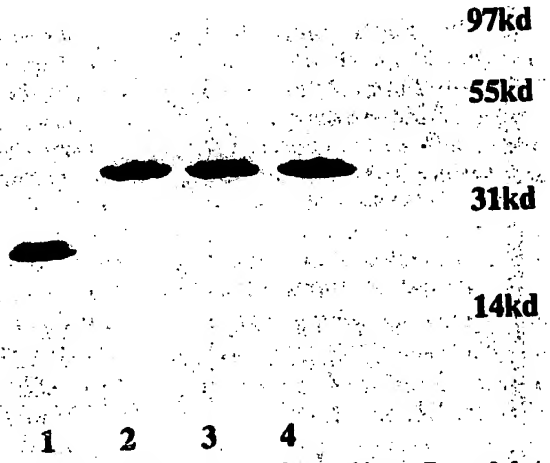

Fig. 3. SDS–PAGE analysis of recombinant Der p 2 fusion proteins. Equal volumes (1 μl) of GST and 3 rDer p 2 variants, affinity purified from 1 liter bacterial expression cultures, were analysed by SDS–PAGE on an 8–25% gel under reducing conditions. Lane 1: GST; Lane 2: C21S; Lane 3: C73R; Lane 4: C119Y. The nomenclature for the rDer p 2 variants is described in Materials and Methods.

Role of disulfide bonds in maintaining antigenic structure

The role of each of the three disulfide bonds in maintaining the antigenic determinants of Der p 2 was evaluated by generating a panel of independent variants and comparing the variants with rDer p 2 in an inhibition ELISA. The ability of the variants to inhibit binding of mAb to rDer p 2 was determined by comparing the amount of variant or rDer p 2 required to give 50% inhibition of Ab binding (Fig. 4). Thus approximately 15 μg/ml of rDer p 2 gave 50% inhibition of mAb αDpX binding (Fig. 4A, 4C, 4E). The C8G variant (shown in Fig. 4A), C21S and C27G variants (Fig. 4C) showed comparable reactivity with αDpX, h wever, the C73R and C78G variants gave <20% inhibiti n of αDpX bind-

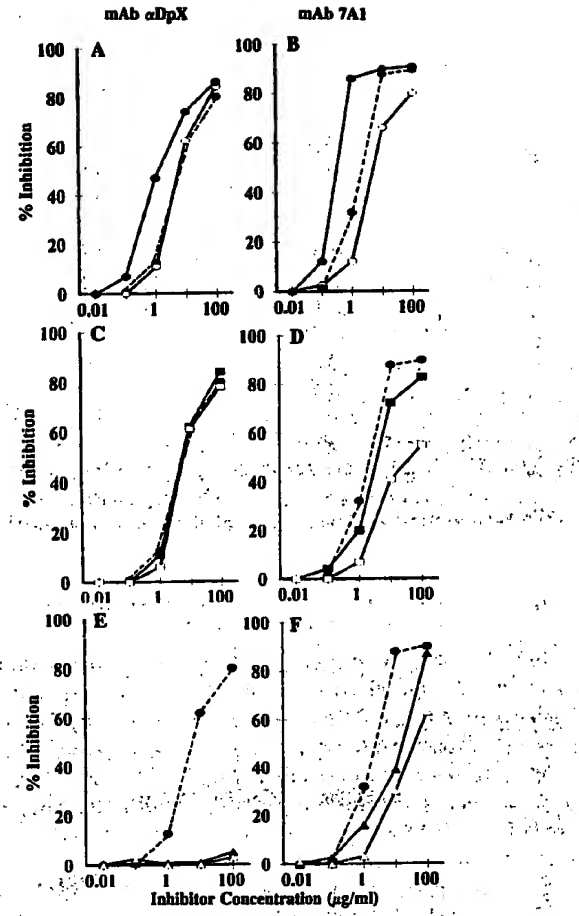

Fig. 4. Monoclonal antibody binding to rDer p 2 cysteine variants. Increasing concentrations of rDer p 2 or rDer p 2 variants were used to block binding of mAb αDpX (Panels A, C and E) and mAb 7A1 (Panels B, D and F) to rDer p 2 in a modified ELISA. Inhibitor antigens were: rDer p 2 (● dashed line), C8G (○) and C119Y (◒) (Panels A and B); C21S (■) and C27G (□) (Panels C and D); C73R (▲) and C78G (△) (Panels E and F).

ing at the highest inhibitor concentration tested (Fig. 4E). The C119Y variant showed increased reactivity for both mAb αDpX and 7A1, giving 50% inhibition at 10-fold less antigen, when compared with rDer p 2 (Fig. 4A and 4B). The mAb 7A1 reacted with the other five variants; the C8G variant (Fig. 4B) and the C21S and C27G variants (Fig. 4D) required 0.5 to 10-fold more antigen to give 50% inhibition, the C73R and C78G variants required 10 to 15-fold more antigen to give 50% inhibition (Fig. 4F). The C73R variant also failed to inhibit the binding of two additional mAb, 13A4 and 15E11 (data not shown). As would be expected for mAb with different fine specificities, all 4 mAb gave unique binding profiles, however, the C73R and C78G variants consistently showed the greatest reduction in antigenicity.

The inhibition of binding f IgE Ab was assessed for 13 individual sera and for the serum pool, from seven additional patients. Using the serum p l, the C8G and C119Y variants gave 50% inhibition of IgE binding at 3-

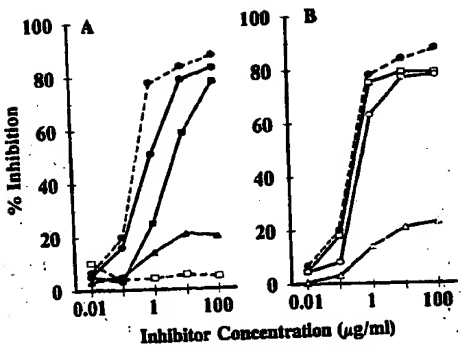

Fig. 5. Inhibition of IgE Ab binding to rDer p 2 using the cysteine variants. rDer p 2 or rDer p 2 variants were used to block binding of IgE Ab from a serum pool as described for Figure 4. The rDer p 2 inhibition curve is shown by (● dashed line). Panel A gives results for inhibitor antigens GST (□ dashed line), C119Y (●), C21S (■), and C73R (▲). Panel B shows results for inhibitor antigens C8G (○), C27G (□) and C78G (△).

to 8-fold higher concentration than rDer p 2; the C21S variant required 35- to 40-fold more antigen, however, the C27G variant gave overlapping inhibition curves with rDer p 2; and the C73R and C78G variants failed to inhibit IgE Ab (Fig. 5). Figure 6 shows the inhibition curves for 4 representative sera of the 13 individual sera

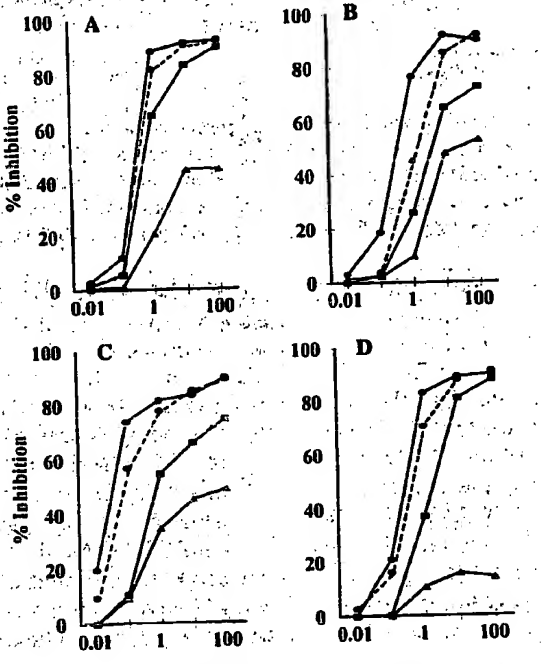

Fig. 6. Inhibition of IgE Ab binding from patients with atopic dermatitis or asthma. Binding of IgE Ab to rDer p 2 was inhibited using rDer p 2 (● dashed line), C119Y (●); C21S (■); C73R (▲). Results are shown for four selected sera: patients J.E. and L.W., shown in Panels A and B, respectively, have atopic dermatitis. Patients K.D. and K.S., shown in Panels C and D, respectively, have asthma. In the absence of inhibitor, using PBS as a control, maximum binding in the ELISA system was OD_{405} = 1.4–1.6, using 1/4–1/16 serum dilutions.

Table 2. Effect of cysteine variants on skin test reactivity

Patient	Quantitative intradermal skin test endpoint (μg/ml) rDer p 2	C78G
M.H.	10^{-1}	10^{0}
M.C.	10^{-2}	10^{-1}
G.R.	10^{-2}	10^{0}
J.L.	10^{-2}	10^{-1}
Control		
T.P.	$>10^{0}$	$>10^{0}$
J.S.	$>10^{0}$	$>10^{0}$
A.S.	$>10^{0}$	$>10^{0}$

Values represent end point dilutions which gave a >8 × 8 mm wheal at 15 min. Negative skin tests are recorded as $>10^{0}$.

tested; Panels 6A and 6B are patients with atopic dermatitis and Panels 6C and 6D are patients with asthma. All of the individual sera were inhibited by C119Y at concentrations equivalent or less than rDer p2. The C21S variant gave overlapping inhibition curves with rDer p2 for two sera, but required 2- to 11-fold more antigen for the other 11 sera. Maximum inhibition by C73R ranged from 11% to 48% for 12 sera and gave 50% inhibition of binding of only one sample at 15 μg/ml, a 12.5-fold concentration increase over rDer p 2. Six sera were from patients with atopic dermatitis and seven were from asthmatics, however, no significant differences in inhibition patterns by the variants were seen among these patients. All 13 sera tested gave the same pattern as seen with the mAb binding to the disulfide variants: the C73R showed the greatest reduction in antigenicity, the C21S variant showed slightly reduced antigenicity, and the C119Y variant showed reactivity comparable to rDer p 2.

Effect of cysteine variants on biological activity of Der p 2

To evaluate *in vivo* reactivity of the variants, quantitative intradermal skin testing was performed using rDer p 2 and the C78G variant. Patients with allergic rhinitis or asthma and Der p 2 specific IgE Ab titers of 40–450 U/ml were tested by prick test using allergen solution of 5 μg/ml. The four patients gave positive prick tests and three non-allergic controls did not react (data not shown). Subjects were injected intradermally with 0.03ml of dilutions of rDer p 2 and C78G. Reactivity to C78G was 10-fold less for three patients and 100-fold less for one patient (Table 2).

DISCUSSION

In 1990, Chua and co-workers cloned the cDNA for Der p 2 and demonstrated that the recombinant protein retained IgE Ab binding reactivity using a solid phase plaque immunoassay (Chua *et al.*, 1990a, 1990b). In the present study, we confirmed that rDer p 2 retains the antigenic structure of the native allergen, by quantitative IgE Ab binding studies using a large panel of sera fr m atopic dermatitis and asthma patients. In additi n, rDer p 2 was rec gnized by murine mAb raised to the native

allergen. These results indicate that the recombinant allergen is an appropriate model system for investigating the antigen structure of Der p 2.

The tertiary structure of Der p 2 has not yet been determined and previous studies in our laboratory suggested that the intact structure, stabilized by three disulfide bonds, was essential for antibody binding (Lombardero *et al.*, 1990). The three disulfide bonds of Der p 2 are similar to disulfide bonds in other proteins. The C21–C27 bond and the C73–C78 bond stabilize small loops that, by analogy to loops in proteins of known structure such as hen egg white lysozyme, are predicted to occur on the protein surface. The C8–C119 bond of Der p 2 is a non-local disulfide bond, separated by 110 residues. Such bonds link the N and C termini in approximately one third of known structures, suggesting this is a good mechanism for stabilizing the folded protein (Thornton, 1981). Using our mutagenesis strategy, one cysteine from each pair was replaced by another amino acid, preventing the disulfide from forming at that position. For this analysis, a variant at each disulfide bonding pair was compared to control for the influence of the side chain substitution.

As would be expected with mAb to independent epitopes, the cysteine variants showed different patterns of reactivity with each mAb. The C73R variant showed the least reactivity with all mAb suggesting that the changes in antigenic structure induced by this variant are more global rather than local to a particular epitope. However, the mAb 7A1 recognized the C73R and C78G variants but failed to bind reduced and alkylated Ag, indicating that these variants are not completely denatured.

Polyclonal IgE Ab binding to the cysteine variants showed a different pattern of reactivity with each protein, however, even in this case, the C73–C78 bond made the greatest contribution to maintaining the antigenic structure of Der p 2, with loss of this bond shifting the inhibition curves by more than 100-fold, illustrated in Figs 5 and 6, and reducing *in vivo* reactivity by 10 to 100-fold, as shown in Table 2. The disruption of the N–C-terminal disulfide shifted the inhibition curve for IgE Ab by 8–10 fold using the serum pool (Fig. 5), but had little effect on the majority of sera and 3 of 4 mAb (Figs 6 and 4, respectively), indicating that the overall conformation of the protein is largely intact. It has been shown that the N–C disulfide of lysozyme is exposed and can be reduced without destroying the structure of the protein (Thornton, 1981). The striking difference between disruption of the C21–C27 and C73–C78 loops may be explained by the C73–C78 bond making the greatest overall contribution to the stability of the protein. Additional changes in antigenicity may reflect the actual substitutions at these positions. However, the reduction in antigenicity for both the C73R and the C78G variants suggests that the loss in activity is directly related to the loss of the bond rather than the amino acid substitutions. The importance of the C73–C78 bond is also supported by studies using synthetic peptides, which showed that the only peptide that bound IgE Ab encompassed residues 73–78 (peptide 65–78) (van't H f *et al.*, 1991). This peptide 65–78 may assume a conformation with the intact disulfide bond, thus mimicking the conformation of this sequence in the native protein. An additional study using serine and methionine substitutions for cysteine in the peptide 65–78 suggests heterogeneity of binding specificities even from an individual donor (van't Hof *et al.*, 1993)

In summary, this study demonstrates the contribution of the three disulfide bonds of Der p 2 to the antigenic structure of the protein. In addition, the bonds make different contributions to maintaining this structure as shown by the IgE Ab binding curves for each variant and the reductions seen *in vivo* on quantitative skin testing. The 10- to 100-fold loss in skin test reactivity observed in these preliminary studies is remarkable for a single amino acid substitution and suggests that targeted substitutions (of Cys or other residues) could result in further reduction of IgE antibody binding. Variants with reduced IgE binding, such as C73R and C78G, may provide an alternative strategy for immunotherapy. Current clinical trials of peptide based immunotherapy use a limited number of allergen specific T cell epitopes to induce T cell unresponsiveness and have yet to establish whether this approach will be sufficient to down regulate T cell responses to the complete allergen and reduce symptoms (O'Hehir *et al.*, 1991; Wallner and Geftner, 1994; van Neerven *et al.*, 1994). The advantage to peptide based therapy is that peptides can be chemically produced in gram quantities and they would be predicted to cause a lower frequency of adverse reactions (through lack of binding to IgE Ab). The disadvantages include rapid metabolism of peptides, the difficulty in designing peptides to bind all HLA alleles, and to cover all immunodominant regions. The use of entire recombinant proteins with reduced IgE Ab binding may offer an alternative approach to immunotherapy, which could potentially be applicable to any cloned allergen.

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Epitope mapping of the house-dust-mite allergen Der p 2 by means of site-directed mutagenesis

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Recombinant Der p 2, expressed in the baker's yeast *Saccharomyces cerevisiae*, was used as a tool to determine IgE- and monoclonal antibody (mAb)-binding sites on this allergen. For this purpose, mutant molecules were produced by application of site-directed mutagenesis. The amino-acid residues spanning cys21–cys27 and cys73–cys78 were deleted, thus preventing loop formation through disulfide bonds. Charged residues in three predicted antigenic sites (residues 45–48, 67+69, and 88–90) were replaced by alanine residues. IgE- and mAb reactivity to these mutants was compared to that to "wild type" Der p 2. Residues spanning cys73–cys78 were involved in the antigenic binding site for mAb αDpX. Mutations in the areas adjacent to this loop (i.e., 67+69 and 88–90) had similar effects on this mAb (10- to 20-fold decreases in reactivity were observed), supporting the suggestion that these areas are involved in this antigenic structure. The area of residues 45–48 was shown to be involved in an epitope for mAb 2B12. The reactivity of mAb 7A1 was influenced by substitutions of residues 45–48 as well as 88–90. Deletion of the residues spanning cys21–cys27 resulted in decreased reactivity to three mAbs (10E11, αDpX, and 7A1). From these observations, it may be concluded that binding of different mAbs is influenced by the same mutations and that the binding of single mAbs is influenced by two or more mutations scattered over the allergen molecule. These findings can point in two directions: minor amino-acid changes result in disruption of the overall conformation of the allergen, or distant sites are close together in the three-dimensional structure of the allergen. Decreased IgE reactivity was observed with all mutant molecules, varying between patients. The observed effects ranged from 5- to 1000-fold. Deletion of the amino-acid residues spanning cys21–cys27 and cys73–cys78 had the strongest effect on IgE reactivity, where decreases up to 1000-fold were observed. Such mutants might be useful tools to improve the safety of allergen-specific immunotherapy.

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Key words: allergen; antigenic binding sites; baker's yeast; house-dust mite; IgE; monoclonal antibodies; recombinant Der p 2; *Saccharomyces cerevisiae*; site-directed mutagenesis.

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The house-dust-mite allergen Der p 2 is a protein of 129 amino-acid residues. The cDNA was originally isolated by Chua et al. (1). Der p 2 contains six cysteine residues which form three disulfide bridges (2), resulting in two small loops and a close link between the N- and the C-terminus.

Epitope mapping of major allergens is a first step in the development of safer immunotherapeutic allergen preparations with low IgE-binding capacity. Several attempts have been made to map epitopes on Der p 2. Human IgE did not show reactivity to peptides (20–30 amino-acid residues) expressed in *E. coli* from a random fragment Der p 2 cDNA library, and recombinant peptides comprising half the linear sequence showed only weak binding to IgE antibodies (3). Another approach was performed in our laboratory by

Abbreviations. α-gal: α-galactosidase; mAb: monoclonal antibody; PBS: phosphate-buffered saline; PBS-T: PBS+0.1% (v/v) Tween-20; PBS-AT: PBS-T+0.3% (w/v) BSA; RAST: radioallergosorbent test; rec: recombinant; RU: RAST unit; WT: wild type.

means of synthetic peptides (4). Whereas this method was demonstrated to be an effective tool in epitope-mapping studies on various allergens, such as Amb a 3 (5), Chi t 1 (6), Gad c 1 (7), Fel d 1 (8), Cor a 1, and Bet v 1 (9), it was less useful for Der p 2. Very few patients had appreciable amounts of IgE against only one peptide (residues 65–78) from a panel of overlapping peptides, spanning the complete Der p 2 molecule (4). Furthermore, from a panel of Der p 2-specific mAbs, none showed reactivity to these peptides (van 't Hof, personal communication). These experimental data agree with the finding that the B-cell epitopes on the Der p 2 allergen are destroyed upon reduction and alkylation (10). Therefore, it is likely that the B-cell epitopes on the group 2 allergen are highly dependent on its conformation, making these techniques for epitope mapping less efficient.

Our approach to investigate B-cell epitopes of Der p 2 was to construct mutants of a yeast-derived recombinant Der p 2 by means of site-directed mutagenesis. The nonmutated, or "wild-type" (WT), Der p 2 has been shown to be almost indistinguishable from natural Der p 2 (11). In two recent reports with a similar approach, the integrity of the three disulfide bridges has been shown to be of importance for IgE and monoclonal antibody (mAb) binding (12, 13). In the present study, the two small cysteine loops were deleted. In addition, several mutations were introduced in potential antigenic sites, selected on the basis of hydrophilicity and surface probability in combination with secondary-structure and backbone-flexibility predictions (14–20).

Herein we describe the production and isolation of these Der p 2 variants from yeast and their reactivity to IgE and mAbs compared to the nonmutated Der p 2.

Material and methods

Sera

Sera (n=20) were collected from subjects showing IgE reactivity (RAST units >0.3) to Der p 2.

Monoclonal antibodies

The anti-Der p 2 mAbs used were αDpX (21), 10E11 obtained after immunization of BALB/b mice with a fraction from mite whole-body extract <60 kDa (van Leeuwen & Aalberse, unpublished), 2B12B3 (22), and 7A1 (23). For application in RAST, ascites or culture supernatants were used. mAbs 10E11 (culture supernatant) and αDpX (ascites) were also protein A purified. αDpX was bioinylated, 0.8 mg NHS-LC-biotin (Pierce, Rockford, IL, USA)/1.2 mg mAb.

Molecular cloning

Molecular cloning of Der p 2 was performed as described elsewhere (11). The Der p 2-containing plasmid pUC-p2 (1) was kindly provided by Dr W. R. Thomas, Subiaco, Australia.

Mutagenesis of the Der p 2 cDNA

Mutations of the Der p 2 cDNA were generated by means of pSELECT™ (Promega, Madison, WI, USA) with a few modifications. The 594 bp *Eco* R I fragment from pUC9-p2 was isolated and ligated into pSELECT, resulting in pSELECT-p2. The single-stranded DNA was isolated by means of infecting the pSELECT-p2 transformant with helper phage M13K07. The *in vitro* mutagenesis was carried out as described in the manual. The putative mutant pSELECT-p2 DNA was isolated from *E. coli* BMH 71-18mutS and hence transformed to *E. coli* JM 110. The transformants were not plated out, but grown in 250 ml 2 YT with ampicillin, and hence the plasmid DNA, was isolated. From this DNA, the 520 bp *Bcl* I–*Hin* d III fragment was isolated. This fragment was ligated into the expression vector pSAY1 (11) digested with *Bcl* I and *Hin* d III. These pSAY1-p2 constructs were then screened by sequence analysis. The oligonucleotides used in this procedure, and the resulting amino-acid changes are listed in Table 1.

Production of recombinant Der p 2 allergens from yeast

Yeast strain *Saccharomyces cerevisiae* BJ 1991 (*MATα, leu2-1, trp1-1, ura3-52, prb1-1122, pep4-3*) (24) was transformed (25) with the appropriate plasmids, and transformants were selected on minimal plates (0.67% yeast nitrogen base without amino acids, 2% glucose, 20 μg/ml uracil, 20 μg/ml tryptophane, and 2.2% agar). The transformants were grown for 40 h in minimal medium; subsequently, the yeast culture was diluted in a 10-fold of this volume in minimal medium with galactose instead of glucose, all at 30°C. After 1 week of growth, the cells were spun down, and the growth medium was further analyzed.

Purification and quantification of Der p 2

A volume of 500 ml growth culture – containing WT rec Der p 2 – was concentrated on an Amicon ultrafiltration cell over a diaflo Ultrafiltration

Table 1. Oligonucleotides used for mutagenesis of Der p 2. Sequences of oligonucleotides used for mutagenesis of Der p 2 cDNA are given with their resulting substitutions in protein. Mutated nucleotides are depicted in both italic and lower case. Deletions in cDNA are represented as –

	Oligonucleotide	Mutation in protein
M45–48	GCCGTTTTCGAAGCCAAC*gcAgcCgCAg* *c*AACGGCTAAAATTGAAATCAAAGCC	gln45.asn46.thr47.lys48 Ø ala.ala.ala.ala
M67+69	GAAGTTGATGTTCCCG*cTATCG cTCCAA* *ATGCATGCCATTACATG*	gly67 Ø ala; asp69 Ø ala
M73–78	GGTATCGATCCAAATGCA*gc–* *cgc*CCCATTTGTTAAAGG ACAACAATATG	cys73 Ø ala, cys78 Ø ala; Δ 74–77
M88–90	GGACAACAATATGAT*gcTgcAgc*TACAT GGAATGTTCCG	ile88.lys89.tyr90 Ø ala.ala.ala
M22–26	CAAAAAAGTTTTGGTACCAGGATGC– TGTATCATTCAT CGTGGTAAACC	Δ 22–26

membrane YM10 (Amicon Corp, Danvers, MA, USA) to a volume of 10 ml. A volume of 1 ml of concentrate was applied to a Superdex 75 HR10/30 column (Pharmacia Biotech, Uppsala, Sweden), equilibrated in 0.05 M $NaPO_4$/0.5 M NaCl/pH 7. The bed volume was 23 ml, and the flow rate was 0.5 ml/min. Der p 2 eluted at between 29 and 36 min (14.5 and 18 ml), resulting in a purity >95%, as judged by SDS–PAGE/silver staining (not shown). The protein concentration of this purified allergen was measured with BCA protein assay reagent (Pierce, Rockford, IL, USA) at 37°C, according to the manufacturer's instructions. BSA was used as protein standard. From this purified Der p 2, a standard was prepared in ×50 concentrated pSY1 growth culture, containing 106 µg Der p 2/ml. This standard plus three consecutive threefold dilutions, together with samples of concentrated growth media containing mutant Der p 2, were electrophoresed on an ExcelGel™ SDS gradient 8–18 (Pharmacia Biotech, Uppsala, Sweden) and silver-stained according to the manual of the producer. The intensities of the Der p 2 bands were scanned with MacImage™ (Xerox Imaging Systems, Inc., Sunnyvale, CA, USA). From the Der p 2 standard curve, the concentration of the allergen mutants in culture supernatants was calculated by Slide Write Plus 6.0.

Der p 2 two-site ELISA

A two-site ELISA for Der p 2 was performed as described previously (11). For quantification purposes, standards were prepared from purified recombinant Der p 2 with known protein content (BCA, Pierce).

Preparation of the solid-phase antigen, RAST, and RAST inhibition

Concentrated (×50) growth medium (300–500 µl) containing 50 µg allergen was coupled to 300 mg CNBr-activated Sepharose-4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's manual. The Sepharose was suspended in 50 ml of PBS/0.3% BSA/0.1% Tween-20 (PBS-T). According to the same procedure, 500 µl concentrated (×50) pSY1 growth medium was coupled to 300 mg Sepharose. The pSY1 growth culture was used as a negative control.

For the RAST, 500 µl (1 mg) Sepharose was incubated with 50 µl antibody sample overnight, end over end, at room temperature. After washing five times with PBS-T, an ^{125}I-radiolabeled antibody against mouse IgG (M1482,CLB) or human IgE (SH25-1-P7,CLB; affinity-purified before iodination) was added. After incubation overnight, end over end at room temperature, the Sepharose was washed four times with PBS-T and the gamma radiation was counted. Results were expressed as percent binding of total counts added.

For RAST inhibition, 50 µl of patient serum or mAb (diluted when necessary, to achieve nonsaturating conditions) was preincubated with 50 µl inhibitor. After 2 h of incubation at room temperature, 500 µl of Sepharose was added, and the RAST was further carried out as described above. The relative inhibitory potency (i.p.) of a particular mutant is defined as the ratio of the amounts of allergens required (rec WT Der p 2/mutant) for 50% inhibition of the RAST.

Results

Mutagenesis of Der p 2 cDNA

The efficiency of the mutagenesis was between 20% and 100% mutant constructs (four to 10 clones were analyzed for each mutation). Loops between cysteine residues 21–27 and 73–78 were deleted (Fig. 1: M22–26 and M73–78), and charged amino-acid residues in three regions (Fig. 1: M45–48, M67+69 and M88–90) were replaced by alanine residues.

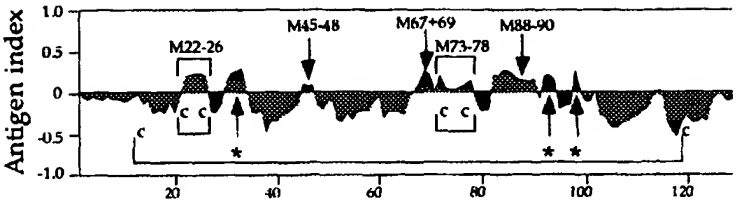

Fig. 1. Antigenicity plot of Der p 2 was derived from mature Der p 2 sequence (Chua et al. [1]) by means of MacVector™ (IBI-A Kodak, New Haven, CT, USA). Codes of mutants are represented. Substitutions and deletions are listed in Table 1. Asterisks refer to three other mutations; namely, arg31.agly32.lys33Ø ala.ala.ala, pro99 Ø ala, and asn93 Ø ala. Cysteine residues are indicated (c) as well as disulfide bonds between them.

In addition, charged residues in three other regions were replaced by alanine residues (namely, arg31.gly32.lys33Øala.ala.ala, pro99Øala, and asn93Øala, represented in Fig. 1 with asterisks). Since these substitutions did not result in a significant decrease of antibody binding in our hands, they are not discussed below.

Expression and quantification of the allergens

The (mutant) Der p 2 constructs were all transformed to yeast, and (mutant) allergen was harvested. Concentrated allergen-containing growth media were separated on SDS–PAGE, followed by silver staining (not shown). The intensities of the rec WT Der p 2 standard and rec WT Der p 2 growth culture were scanned. By this procedure, the concentration of Der p 2 in concentrated growth culture was calculated by linear regression: 140 µg/ml. This value was in good agreement with quantification in a two-site ELISA (150 µg/ml). The growth culture of all Der p 2 variants was also scanned, and the concentrations were calculated similarly (Table 2).

Table 2. Expression of Der p 2 and Der p 2 mutants. Scanned areas from Der p 2 standard, and allergens in culture supernatants are given. From plot of standard curve, concentrations were calculated. Dilution of allergen sample is represented.

Sample	Scanned area	Dilution	Concentration µg/ml
Der p 2	714539	1	106
Standard	524291	1	35
	377497	1	12
	304622	1	4
Der p 2	593911	2	140
M45–48	561404	1	61
M67+69	602394	1	77
M73–78	469320	2	66
M22–26	314853	1	6
M88–90	582888	2	138
α-gal	208286	1	—

Reactivity to mAbs

The binding to the five Der p 2 mutants was studied with four different mAbs in RAST (Table 3). In addition, RAST inhibitions were performed with mAbs 2B12, αDpX, and 10E11 and mutants M45–48, M67+69, and M73–78 as inhibitor (Fig. 2 and Table 4).

2B12B3

Substitutions in the area 45–48 (M45–48) resulted in a decrease in reactivity of approx. 300-fold with mAb 2B12B3, as judged by RAST inhibition. Deletion of the stretch of residues at 73–78 (M73–78) had a minor effect on the binding of this mAb: approx. fivefold reduction (Fig. 2A). The other three mutants studied did not show significant changes in binding to mAb 2B12B3.

αDpX

Substitution of residues 67 and 69 (M67+69) or deletion of the adjacent stretch of residues 73–78 (M73–78) had similar effects on the reactivity of mAb αDpX in RAST inhibitions, indicating that the area at residues 65–80 is crucial for the integrity

Table 3. Reactivity of Der p 2 mutants to different mAbs. Der p 2 RAST for four mAbs was carried out with Der p 2 and Der p 2 mutants. Sepharose was diluted five times in glycine Sepharose in PBS-AT. Dilution of mAbs were 7A1 40000 (ascites); 2B12B3 81000; and 10E11 20 and αDpX 10000 (culture supernatants) times. Results are expressed as percentages of added counts

	2B12	αDpX	7A1	10E11
α-gal	0.4	0.8	0.5	0.4
Der p 2	43.2	47.3	18.1	37
M45–48	6.3	13.5	4.0	10.9
M73–78	26.9	9.3	20.3	6.4
M88–90	45.2	12.8	10.7	40.2
M22–26*	42.2	6.0	1.9	1.5
M67+69	42.9	4.3	19.6	25.9

*For M22–26, due to low expression level, 30 µg, instead of 50 µg, was coupled.

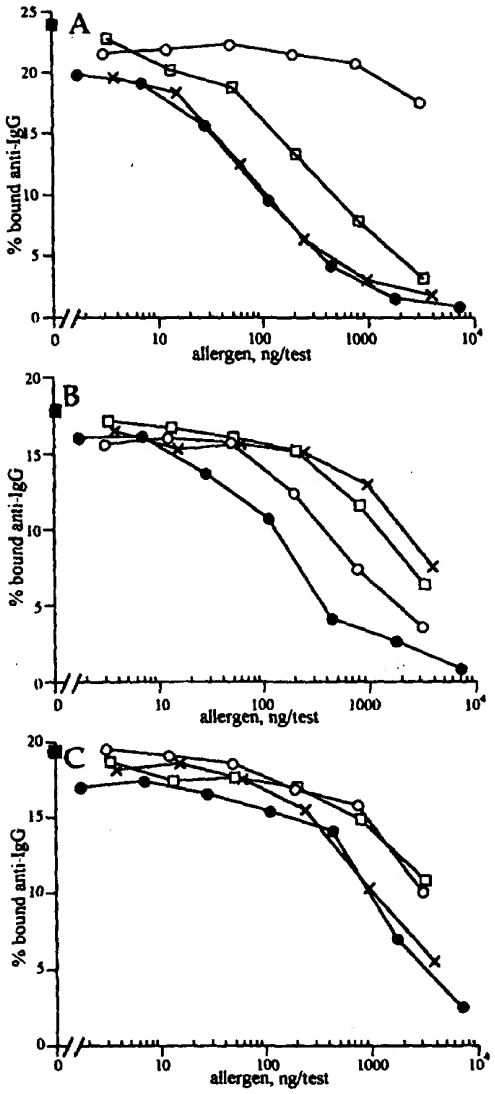

Fig. 2. RAST inhibition for mAbs. RAST inhibitions were carried out with (rec) Der p 2 on Sepharose. RASTs were inhibited with (rec) Der p 2 and Der p 2 mutants. Results are represented as percentage of added counts. Uninhibited RAST values are indicated by ■. A) mAb 2B12B3; B) mAb αDpX; C) mAb 10E11. ●=Der p 2, ○=M45–48, X=M67+69, □=M73–78.

of the epitope for αDpX. Mutants M67+69 and M73–78 were shown to be approx. 30- and 10-fold weaker inhibitors of the WT Der p 2 RAST, respectively (Fig. 2B). Even replacement of three charged residues a little further downstream of the C terminus (M88–90) still affected the binding of this mAb (Table 3). M45–48 had a minor effect – approx. three times weaker inhibitor – although it is located far from the area around the disulfide bond cys73–78 (Fig. 2B). However, it cannot be excluded that this effect falls within the margins of accuracy of the protein-quantification method for the mutants. Finally, the deletion of amino-acid residues 22–26 (M22–26) resulted in significantly decreased binding of mAb αDpX (Table 3).

7A1

Mutations in the areas 45–48 (M45–48) and 88–90 (M88–90) had a significant effect on reactivity to mAb 7A1 (Table 3). M22–26 was no longer recognized by mAb 7A1. The mutants M73–48 and M67+69 did not show altered reactivity.

10E11

A previous study showed that addition of one alanine residue to the N-terminus had a drastic effect on the binding of mAb 10E11 (Hakkaart et al., paper submitted). The RAST data obtained in the present study indicate that mutants M67+69, M73–78, M45–48, and M22–26 show decreased reactivity to this mAb. In RAST-inhibition assays, no difference between WT Der p 2 and M67+69 was observed (Fig. 2C). For M45–48 and M73–78, inhibition curves shifted by a factor of 3. Therefore, as for mAbs αDpX and M88–90, it cannot be excluded that these differences are explained by inaccuracy in quantification of the mutants.

Reactivity to human IgE

Direct RAST. For 20 sera, RASTs were carried out with rec WT Der p 2 and mutants on Sepharose. At saturating solid-phase allergen concentrations, the ratios of IgE binding (mutant/WT) were in most cases >0.4. For M45–48, there were three exceptions (0.39, 0.22, and 0.36); for M73–78, two (0.36 and 0.38). Only for M22–26 did most sera (15/20) demonstrate lower IgE binding.

RAST inhibitions. For six of these sera, RAST inhibitions were carried out. For a selection of these experiments, inhibition curves are shown in Fig. 3A–C. The inhibitory potencies of the mutants, relative to WT Der p 2, are shown in Table 4 for all six sera analyzed.

M45–48: gln45.asn46.thr47.lys48 Øala.ala.ala.ala. When charged residues at positions 45–48 were replaced by alanines, decreases in inhibitory potency varied from 3 to 100 times (Table 4). In Fig. 3A, inhibition curves are shown for serum 46 and serum CV. Approximately 30 times more of mutant M45–48 was needed for inhibition in the case of the latter serum.

Table 4. Relative inhibitory potencies of Der p 2 mutants. For three mAbs and six sera, rec WT Der p 2 RAST was inhibited with rec WT Der p 2 and mutants. Inhibitory potency for mutant is given as ratio of amounts of allergen required (rec WT Der p 2/mutant) for 50% inhibition of RAST

	Monoclonal antibodies			Sera					
	2B12B3	aDpX	10E11	2	46	135	136	CV	MV
M45–48	0.03	0.33	0.34	0.21	0.29	0.34	0.20	0.009	0.17
M67+69	0.90	0.057	0.44	0.072	0.88	0.004	0.018	0.18	0.91
M73–78	0.20	0.11	0.30	0.10	0.57	0.17	0.080	0.009	0.12
M22–26	Nt	Nt	Nt	0.001	0.002	0.14	0.007	0.002	0.07
M88–90	Nt	Nt	Nt	0.015	0.17	0.28	0.080	0.039	0.13

Nt: not tested.

M67+69: gly67 Ø ala; asp69 Ø ala. Two out of six sera tested in RAST inhibition showed an IgE reactivity of M67+69, comparable to that of Der p 2 (serum 46 and MV). For the other four sera, the decrease in reactivity was 5- to 250-fold (Table 4). Inhibition curves for a nondiscriminating serum (MV) and for a serum with a 250-times decreased reactivity (serum 135) are shown in Fig. 3B.

M73–78: cys73 Ø ala; cys78 Ø ala; Δ 74–77. In mutant M73–78, the cysteine loop at this position was deleted. This deletion resulted in decreases of reactivity of up to 100-fold (Table 4) observed for serum CV. IgE binding of serum 46 was hardly affected.

M22–26: Δ 22–26. In mutant M22–26, the amino acids between cys21 and cys27 were deleted, avoiding formation of the disulfide bridge between the cysteines. In the RAST inhibitions for the six sera tested, decreases in IgE reactivity ranged from 10- to 1000-fold (Table 4). Two extremes (serum 2 and 135) are shown in Fig. 3C.

M88–90: ile88.lys89.tyr90 Ø ala.ala.ala. Replacing amino acids 88–90 by alanines resulted in 3- and 65-fold decreases in IgE reactivity (Table 4). IgE binding of serum 2 was most affected.

Discussion

In this study, rec Der p 2 mutants and rec "WT" Der p 2 were compared with regard to their reactivity to mAbs and IgE in order to identify antigenic binding sites.

A problem in this study was the quantification of each mutant in yeast culture supernatants. Quantification is ideally carried out with monospecific antibodies. However, these antibodies behave differently with each mutant. Therefore, quantification was performed by an electrophoretic method (SDS–PAGE) in conjunction with silver staining and scanning. The accuracy of this method is not extremely high. This indicates that in the worst case the highest relative inhibitory potency observed with a mutant represents the lowest estimate in quantification. If we keep this in mind, all mutations resulted in a significant decrease in reactivity to mAb and IgE, ranging roughly from 5- to 1000-fold.

A significant decrease in antigenicity was observed in M67+69, thus indicating substitution of amino-acid residues 67 and 69. This led to a decrease in reactivity to the mAb αDpX. Deletion of the stretch residues between the two cysteines at positions 73–78 (M73–78) had a similar effect on the reactivity to αDpX. These results suggest that this whole area is involved in an epitope. In the meantime, other studies have confirmed the importance of the disulfide bridge 73–78 in stabilizing the epitope for αDpX (12). In these studies, the role of the disulfide bridges in the Der p 2 molecule was investigated by substitution of the cysteine residues, preventing these bridges from being formed. The disulfide bridge at cys73–cys78 was found to be the most important for IgE binding (12). Other studies, however, have indicated that cys8–cys119 is the most important disulfide bridge in antigen binding (13, 26). In contrast, the first study reported this bridge to be the smallest contributor to antigenicity (12). The discrepancies may be caused by differences in the recombinant Der p 2 (or Der f 2) molecules tested, or may be related to differences in the sera used for the studies.

In our study, however, the largest decrease in IgE reactivity was observed in mutant M22–26; this was caused by the deletion of the stretch of amino-acid residues at cys21–cys27. It cannot be excluded that these amino-acid residues themselves are involved in an antigenic binding site, and that therefore the breaking of the disulfide bridge itself is not enough to cause a large decrease in antigenicity.

Another location found to be involved in an antigenic binding site was the stretch of residues 45–48. Replacement of these residues (M45–48) by alanine residues led to a decrease in reactivity

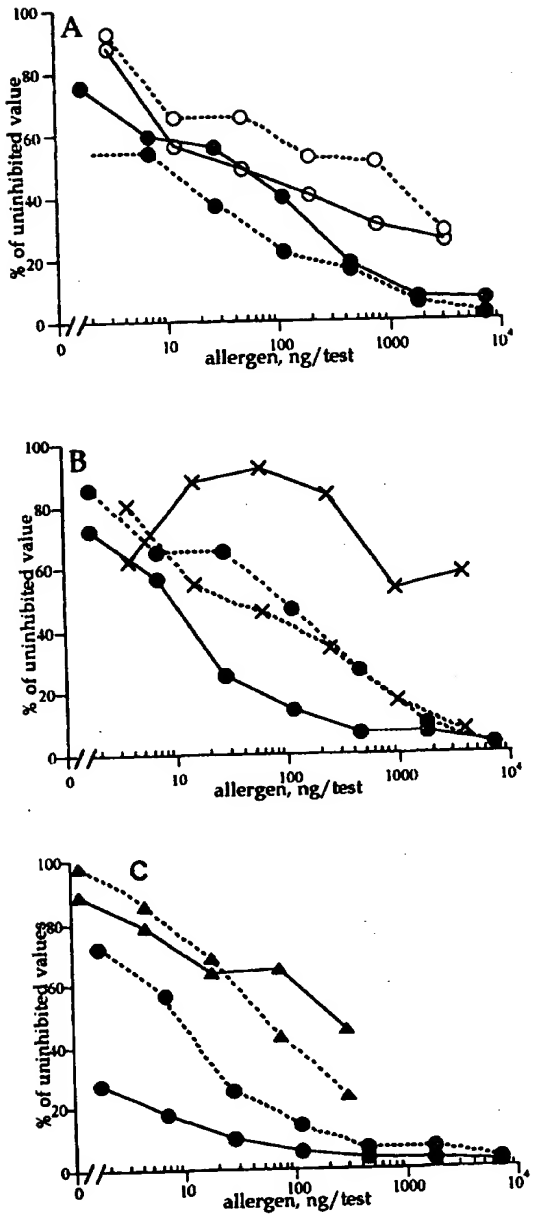

Fig. 3. RAST inhibition for IgE. RAST inhibitions were carried out with rec WT Der p 2 on Sepharose. RASTs were inhibited with rec WT Der p 2 and Der p 2 mutants. Results are represented as percentage of uninhibited value. ●=Der p 2, O=M45–48, X=M67+69, Δ=M22–26. A) serum 46 (solid line) and serum CV (dashed line) with M45–48; B) serum 135 (solid line) and serum MV (dashed line) with M67+69; C) serum 2 (solid line) and serum 135 (dashed line) with M22–26.

to mAbs 2B12B3 and 7A1. The effect for 2B12B3 was the strongest, approx. a 300-fold decrease in reactivity. In IgE reactivity, a significant decrease could also be demonstrated.

The latter mutation together with mutation M88–90 resulted in a decrease in reactivity to mAb 7A1. The fact that both distance mutations influence the epitope for one mAb may suggest that the peptide sequences are somehow adjacent in the native molecule. This is supported by the finding that M45–48 also influences the reactivity to mAb αDpX. This mAb was shown to be directed to the area around disulfide bridge 73–78 (M67+69/M73–78/M88–90).

Deletion of residues at cys21–cys27 (M22–26) led to a large decrease in antigenicity. For three mAbs, decreased reactivity was observed. However, one mAb (2B12B3) showed strong binding. This might suggest that this mAb shows strong reactivity to partially unfolded Der p 2. The fact that this mAb shows strong reactivity to most of the mutants (except M45–48) points in the same direction. For M22–26, the decrease in IgE reactivity for sera tested was the largest; a decrease in relative inhibitory potency varying from 10- to 1000-fold was observed. The observation that all mAbs – except 2B12B3 – showed a large decrease in binding to M22–26 suggests that the overall conformation is disturbed in this mutant.

Although this approach resulted in the development of a set of Der p 2 variants with diminished antibody binding, some critical comments also have to be made on the antigenicity-prediction method. Amino-acid residues 31–33, 93, and 99 were also replaced by alanine. These variants did not show loss of antigenicity (results not shown), despite the fact that they were predicted. However, the computer predictions achieved with MacVector™ were a useful key in this epitope-mapping study. Random mutagenesis – followed by an effective screening procedure – might provide valuable additional information.

In conclusion, we constructed a set of Der p 2 variants with diminished immunologic reactivity. Preliminary studies indicated that these mutants also possess a decreased ability to induce histamine release from basophilic leukocytes (results not shown). Furthermore, these variants will be applicable in studies on the molecular mechanisms of mast-cell activation. Hence, monospecific IgE can be isolated by means of absorption IgE sera with particular Der p 2 mutant molecules. *In vitro* sensitization of basophilic leukocytes with this IgE (and combinations) may be used to study the minimal requirements needed to trigger the basophilic leukocytes (or mast cells). Furthermore, since all variants (except M22–26) tested still possessed the ability to induce histamine release from basophilic leukocytes, double or triple mutants might be developed from these experimental data, in order to obtain variants that lack biologic activity. Some of these variants may be useful in immunotherapy.

Acknowledgments

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MOLECULAR CHARACTERIZATION OF ALTERNARIA ALTERNATA AND CLADOSPORIUM HERBARUM ALLERGENS

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1. INTRODUCTION

Investigations of fungal air spora have demonstrated that conidium-forming fungi usually predominate over other fungal groups. Cladosporium herbarum, Alternaria alternata, Penicillium sp.and Aspergillus sp., listed in decreasing frequency of occurrence, are most consistently associated with the highest mean percentages of total fungal spore catches. In addition to daily variations in meteorologic conditions and seasonal changes, both of which have enormous impact on the concentration of air spora, the amount and type of vegetation of a region or microenvironment may be important factors in determining the composition of the airborne fungal population. Over the past two decades most allergologists have recognized a need for more information on fungal allergy because of increased awareness of the problem and the greater number of patients suffering from asthma and rhinitis due to fungi. Seasonal variation in regard to fungi in the air or to those in homes, and their detection and monitoring but also the biological relevance of the allergenic protein in the fungal organism are new fields of studies (1, 2). In the environment, Cladosporium herbarum is the most frequently encountered mould in the air. The dry conidia are carried easily through the air and can be detected in extremely large numbers e.g. a concentration of 35000 conidia/cubic meter can often be measured. The indoor counts to a large extent reflect the outdoor concentration. Depending on climate conditions the conidia may begin to appear in the atmosphere in spring and rise to a peak in either late summer or early autumn. Cladosporium herbarum is one of the most common colonizer of dying and dead plants and also occurs in various soil types and on food. It can

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frequently be found in uncleaned refrigerators, foodstuff, on wet window frames, in houses with poor ventilation and houses with straw roofs (3, 4, 5).

Spores of Alternaria alternata are the most important mould allergens in the United states. Alternaria is found predominantly on the East coast, in the upper Midwest and in southern California, probably ranking third only to ragweed and grass pollens as a natural cause of allergy. Known habitats of Alternaria alternata are soils, corn silage, rotten wood, composts and various forest plants. It is frequently found on wet window frames. It is considered an outdoor mould and appears when the weather is warm (3, 4, 5).

One of the most notable properties of fungal antigens is their complexity, which makes great problems for diagnosis. Skin testing is often said to be more sensitive than in vitro testing. At least in the case of mould allergens, such statements can be questioned. It is also a major clinical problem to identify the extent to which symptoms of a mould atopic patient can be attributed to mould allergy. This is because exposure to moulds is a continuum without definite seasonal end-points. Given the importance of the two moulds mentioned above and the unclear situation with respect to classical allergen characterization, it seemed highly desirable to us to clone and sequence cDNAs coding for the major and minor allergens of the two moulds. Cloning, sequencing, and recombinant production of allergens seems to be a prerequisite to improvements in both diagnosis and therapy. We are reporting here ten complete sequences of cDNAs coding for major and minor allergens of Cladosporium herbarum and Alternaria alternata.

2. METHODS

2.1. Biological Materials

Sera from 194 patients allergic to moulds were selected for this study. About 60% of them had positively tested to Alternaria or Cladosporium in RAST, the others had positively tested to a mould allergen mixed RAST (Pharmacia, Uppsala, Sweden). The sera were supplied by three Austrian allergy clinics: i) Allergieambulatorium Reumannplatz, Vienna: 133 sera; ii) Lungenambulanz Krankenhaus Lainz, Vienna: 38 sera; iii) Allergieambulanz Landeskrankenhaus Salzburg: 23 sera. None of the patients had received hyposensitization treatment before testing was performed.

Cladosporium herbarum (strain collection number: 28–0202) was obtained from the microbiological strain collection of the Institute of Botany, University of Regensburg, FRG (courtesy Prof. Helmuth Bezel).

Alternaria alternata was obtained from the Institut für Gärungsgewerbe, Technical University of Berlin (strain collection number: 08–0203, courtesy Prof. Ulf Stahl).

Cultivation of the two moulds was done as described previously (6).

2.2. Procedures

cDNA libraries from Cladosporium herbarum and Alternaria alternata were constructed as described (6). Using serum IgE from Alternaria and Cladosporium allergic patients a series of IgE-binding clones were obtained. Recombinant allergens were then tested as fusion and non-fusion proteins for their IgE reactivity. cDNA clones encoding major and minor allergens were subjected to DNA sequence analysis and the deduced amino acid sequences were compared with the SWISS PROT protein data-base.

Table 1. Summary of major and minor allergens of Alternaria alternata and Cladosporium herbarum recognized by our patients' collective

kD (biological function)	Alternaria alternata			Cladosporium herbarum		
	Allergen	% of patients	Cloned	Allergen	% of patients	Cloned
110 red.	-		-	Cla h 8	11	-
100 red.	-		-	Cla h 9	6	-
85 red.	Alt a 3	42	-	-		-
63 red. (PDI)	Alt a 4	37	+	-		-
53 red. (ALDH)	Alt a 10	2	+	Cla h 6	20	+
48 red. (enolase)	-		-	Cla h 3	40	+
45 red.	Alt a 2	47	-	Cla h 2	43	-
42 red.	Alt a 9	5	-	Cla h 7	17	-
39 red.	Alt a 8	7	-	-		-
30 non-red	Alt a 1	80	-	-		-
30 red.	Alt a 5	33	-	Cla h 1	61	-
22 red. (homolog to YCP4 yeast protein)	Alt a 7	30	+	Cla h 5	22	+
11 red. (P1 ribosomal protein)	Alt a 11		+	Cla h 10	20	+
11 red. (P2 ribosomal protein)	Alt a 6	25	+	Cla h 4	22	+

Summary of all allergens of Alternaria alternata and Cladosporium herbarum that are recognized by our patients. With the exception of Alt a 1, all allergens can be detected in an immune blot under reducing conditions. In total, ten IgE-reactive proteins could be detected in Alternaria, five of which have been cloned. Nine IgE-reactive proteins could be detected in Cladosporium, five of which have been cloned. Five allergens co-migrate and could be closely related in both moulds. Four of them (ALDH, YCP4, P1 and P2) not only co-migrate in Alternaria and Cladosporium, but could be shown by cDNA cloning and sequencing to be very probably functionally closely related.

3. RESULTS

3.1. Analysis of the cDNA Inserts

A total of about 150 positive clones were identified by screening each of the two libraries with a serum mixture of two patients. In the case of Cladosporium herbarum as well as in the case of Alternaria alternata 5 different types of inserts could be detected. In order to confirm that the recombinant phages isolated in this way indeed expressed fusion proteins reactive with the IgE used for screening of the libraries, the fusion proteins were induced, separated by SDS-PAGE and tested in immune blots again. According to our sequencing data one could see that the cloned allergens of Cladosporium herbarum and Alternaria alternata belong to 5 groups of homologous proteins (Table 1): Allergen Alt a 10 (Mr=53 kDa, EMBL accession number: X78227) of Alternaria alternata and Cla h 6 (Mr=53 kDa, EMBL accession number: X78228) of Cladosporium herbarum by comparison with the protein data base were identified as aldehyde dehydrogenases (ALDHs). Aspergillus fumigatus ALDH yielded the highest score of identity and homology.

Allergen Alt a 6 (Mr=11 kDa, EMBL accession number: X78222) of Alternaria alternata, allergen Alt a 11 (Mr=11 kDa, EMBL accession number: X84216) of Alternaria alternata, Cla h 4 (Mr=11 kDa, EMBL accession number: X78223) of Cladosporium her-

barum and Cla h 10 (Mr=11 kDa, EMBL accession number: X85180) of Cladosporium herbarum were identified as ribosomal proteins (P1 and P2). P1 and P2 are members of a family of three eukaryotic acidic ribosomal phosphoproteins (P-proteins), P0, P1 and P2, which are well known lupus erythematodes antigens (7).

Allergens Alt a 7 (Mr=22 kDa , EMBL accession number: X78225) of Alternaria alternata and Cla h 5 (Mr= 22 kDa, EMBL accession number: X78224) of Cladosporium herbarum show significant homologies to the YCP4 gene of Saccharomyces cerevisiae. YCP4 in Saccharomyces cerevisiae was identified by genome sequencing and has no known function. It is a non-essential yeast gene (8). A possible function for the YCP4 gene is indicated by the recent finding that the Schizosaccharomyces pombe homolog of YCP4 confers resistance to brefeldin A. This protein shares 58% homology with Alt a 7 and Cla h 5 (14).

Allergen Alt a 4 (Mr=63kD, EMBL accession number:X84217) of Alternaria alternata shows high homology to PDI (protein disulfide isomerase), a well known enzyme responsible for the proper folding of proteins to their 3-dimensional structure (9).

Allergen Cla h 6 (Mr=47 kDa, EMBL accession number: X78226) of Cladosporium herbarum is an enolase. In Saccharomyces cerevisiae, two very closely related enolases exist, one of which (enolase 1) is a heat shock protein (10). It is impossible to decide by sequence comparison alone whether Cla h 6 is also a heat shock protein. Further experiments are clearly needed to clarify this point .

3.2. Comparison of the Recombinant Allergens with the Native Allergens

The fusion part of the recombinant allergens made it hard to correlate the bands in the Westernblot with the natural allergens. Therefore we cloned all ten cDNAs as recombinant non-fusion allergens in the expression vector pMW172, transformed the recombinant plasmids in the E. coli strain BL21 and induced recombinant allergen synthesis with IPTG. Two interesting questions could be answered by these experiments. First, by alignment with Coomassie-stained SDS-PAGE gels of Alternaria and Cladosporium extracts we could show that the ten cloned allergens are not prominent protein bands in the extracts. Second, exact co-migration and similar IgE binding of recombinant and natural allergens was observed (6). The co-migration indicates the correspondence between cloned allergens and bands in immune blots of fungal raw extracts and very probably means, that the nine allergens we are dealing with here are not postsynthetically modified.

4. DISCUSSION

We are presenting here our successful attempt to characterize the allergens of Alternaria alternata and Cladosporium herbarum by cDNA cloning, sequencing, and expression in E. coli of recombinant non-fusion proteins. This approach seems to be the only one that leads to a reproducible well characterized and pure source of allergens for diagnosis and therapy (2).

The ten new mould allergens presented here represent six new allergenic proteins: ALDH, Enolase, YCP4, PDI and the acidic ribosomal proteins P1 and P2. Four of them (ALDH, YCP4 and P1 as well as P2) were found to be allergens in both fungi, Alternaria and Cladosporium. A comparison of these allergens with the known allergens of pollens, house dust mites and foodstuffs allows for a few generalizations to be made:

1. None of the mould allergens that are now known with respect to sequence and/or enzymatic function corresponds to a previously known non-fungal allergen, although many of the known fungal allergens do occur as homologous allergens in more than one fungal species.
2. We notice that all the newly sequenced fungal allergens presented here are soluble cytoplasmatic proteins (as judged from their function). For comparison, both, soluble cytoplasmatic proteins (11, 12) and secreted proteins have been found among cloned and sequenced pollen allergens. The cytoplasmatic allergens are very probably non-glycosylated. Indeed, the ten allergens presented here were produced as recombinant non-fusion proteins and exhibited exactly the same electrophoretic mobility and IgE-binding properties in Western blots as the natural allergens of the mould extracts.
3. All the allergens presented here are rather highly conserved proteins. At least one of them, enolase, has been shown to be an important allergen of Candida albicans (13). The human P1 and P2 ribosomal proteins are well known lupus erythematodes antigens, the relevant epitope for LE being the highly conserved C-terminal sequence KEESEESDD/EDMGFGLFD. Deletions of the C-terminal part of the P2 sequence showed, that the KEESEE-epitope is not the only IgE binding epitope in the P2 protein.
4. Most of the ten allergens presented here are "household" proteins needed for basic metabolism like glycolysis or protein synthesis. This fact makes it unlikely that these allergenic proteins be spore-specific. However, the fungal material used for the present study contained vegetative cells as well as conidiospores and cells and conidiospores were not separated prior to allergen extraction. Further investigations will show whether some of the not yet cloned and sequenced allergens of Alternaria and Cladosporium are indeed spore-specific.

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Localization of antigenic sites on Der p 2 using oligonucleotide-directed mutagenesis targeted to predicted surface residues

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Summary

Background Understanding the molecular nature of allergen–antibody interactions is important to understanding the mechanism of conventional immunotherapy as well as to designing alternative immunotherapeutic strategies. Many important allergens have been cloned and expressed, making it possible to apply recombinant DNA techniques to dissect antigenic determinants.

Objective The aim of this study was to use predictive algorithms and site-directed mutagenesis to investigate monoclonal antibody and IgE antibody epitopes of the major house dust mite allergen Der p 2.

Methods Computer algorithms were used to assess the primary amino acid sequence of Der p 2 and to identify regions of hydrophilic and flexible sequence. Subsequently, site-directed mutagenesis was used to generate amino acid substitutions at hydrophilic residues at positions 44–46 and at position 100. The variants were tested in a competitive inhibition ELISA with four group 2-specific murine monoclonal antibodies and with human IgE antibody from mite allergic patients.

Results Conservative amino acid substitutions at position 44–46 did not distinguish IgE antibody epitopes, but did suggest that these residues are involved in the epitope defined by one monoclonal antibody, 15E11. Non-conservative substitution of proline at this position reduced binding to all four monoclonal antibodies, as well as IgE antibody, by 50–80%. Point mutants at position 100 mapped the epitopes of two monoclonal antibodies, 7A1 and 13A4, previously shown to bind the same region of Der p 2. In addition, the two variants tested at this position showed distinct inhibition curves with these two monoclonal antibodies indicating differences in fine specificity.

Conclusions Using predictive algorithms, in the absence of tertiary structural information, we have been able to localize important B cell determinants on Der p 2. The results suggest that it is possible to modulate antibody recognition of allergens using site-directed mutagenesis and that this approach may provide a new strategy for allergen specific immunotherapy.

Keywords: allergens, antigenic determinants, dust mites, IgE antibody, monoclonal antibody, mutagenesis, secondary structure

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Introduction

During the past decade, many allergens have been isolated, cloned and sequenced, making it possible to dissect the antigenic structure of proteins that cause immediate hypersensitivity. Knowledge of the molecular nature of allergen-antibody (Ab) [1] interactions is important for designing new and more effective immunotherapeutic strategies for the treatment of allergic disease. B-cell epitope mapping studies most commonly involve functional approaches such as chemical modification of the antigen, binding studies with peptides and proteolytic fragments, and analysis of naturally occurring isoforms and point mutants. Structural analyses using X-ray crystallography and nuclear magnetic resonance spectroscopy are technically more difficult. However, the three dimensional structure has been determined for several antigen-antibody complexes using murine monoclonal antibody (MoAb) in complex with protein antigens such as hen egg lysozyme and staphylococcal nuclease. Combinations of these studies have defined the area of the antigen-Ab interface, the binding energies involved, and the role of affinity maturation in stabilizing the antigen-Ab complex [1–5].

Site-directed mutagenesis has been used successfully for epitope mapping of antigens of known three-dimensional structure, where surface residues are targeted [6–9]. The assumptions in this approach are that altering amino acid side chains that are surface exposed has minimal effect on the tertiary structure of the antigen and that changes in Ab binding occurring as a result of an amino acid substitution indicate the importance of that residue to the epitope.

The group 2 allergens are comprised of a family of isoforms that show extensive antigenic crossreactivity. Genomic and cDNA cloning have demonstrated at least three unique Der p 2 sequences and four Der f 2 sequences [10,11]. Crossreactive and species-specific MoAbs have recently been developed; however, epitope mapping using these MoAbs may be confounded by the fact that the isoforms have 2–10 amino acid differences making it difficult to assign a particular region of the allergen to a particular epitope [12–15]. Investigators have addressed the nature of B- cell epitopes on Der p 2 using large polypeptides generated from cDNA fragments as well as short synthetic peptides spanning the entire primary sequence [16,17]. Both studies showed a very low frequency of IgE Ab binding and concluded that the majority of epitopes on Der p 2 are conformational, rather than sequential, in nature. These conformational determinants are stabilized by the three disulfide bonds and in a previous study we used site-directed mutagenesis to investigate the contribution of each of these bonds to the antigenic structure of Der p 2 [18–20].

In this study, we used predictive algorithms to identify surface residues (potential antigenic sites), since the tertiary structure of Der p 2 was not known. These algorithms have been shown to be useful for predicting antigenic determinants [21–23]. Site-directed mutagenesis was used to generate a panel of variants at these positions, and MoAb and IgE Ab binding to the variants and to recombinant (r) Der p 2 was compared using inhibition ELISA. Using this approach, amino acid residues on Der p 2 that form part of epitopes recognized by MoAb and IgE Ab have been identified.

Materials and methods

Oligonucleotide mutagenesis

The cDNA for Der p 2 was obtained from Dr Wayne Thomas, Perth, Australia, and subcloned into the plasmid M13mp19 [24]. Oligonucleotide directed mutagenesis was performed as previously described [20]. Oligonucleotides used to generate amino acid substitution at positions 44–46 and 100 are shown below: (N indicates a mixture of the four deoxyribonucleotides)

Asp44–46: 5′-GCC GTT TTC GAA GCC NNC/G NNC/G NNC/G ACA AAA ACG GC-3′;

Lys100: 5′-CCG AAA ATT GCA CCA NNC/G TCT GAA AAT GTT GTC G-3′;

Mutants were identified by DNA sequence analysis. The sequences were PCR amplified from the M13 templates using specific 5′ and 3′ terminal primers containing BamH1 and EcoR1 restriction endonuclease sites, respectively. These fragments were subcloned into the BamH1/EcoR1 site of the pGEX2T vector (Pharmacia Biotech) and DNA sequence analysis showed that the sequence in the expression vector was identical to the Der p 2 cDNA except at the targeted codon(s). Each variant was expressed and purified as previously described and protein concentration measured by Bradford Assay [20]. The variants were produced in approximately equal amounts from bacterial expression cultures (1–4 mg/L) and gave a single band of 41 kDa on SDS-PAGE (data not shown).

Production and purification of recombinant (r) Der p 2

The plasmid pGEX2TP2 produces rDer p 2 as a fusion protein with glutathione-S-transferase. The unmodified glutathione-S-transferase (GST) fusion protein used for Ab binding studies was designated rDer p 2; variants were designated by the single letter amino acid abbreviation preceding the position of that residue in the primary sequence, followed by the single letter code for the variant. For example: K100T is the threonine substitution for lysine at position 100. For simplicity, variants at positions 44–46 with multiple substitutions are designated by the substitution at position 44, e.g. N44Q is the substitution of

glutamine, threonine, threonine at positions 44, 45, and 46, respectively.

Secondary structural predictions of Der p 2

Chou Fasman, Neural net, and Garnier-Robson algorithms were used to predict the secondary structure from the primary amino acid sequence deduced from the Der p 2 cDNA [25–27]. The hydrophilicity profile was generated using the Hopp-Woods algorithm, using a window of seven residues, and the flexibility profile was produced with the Karplus and Schultz algorithm [22,23]. All computer aided sequence analysis was performed using a Silcon Graphics work station.

Murine and human Ab to mite allergens

The specificity of the murine MoAb used in this study has been described previously [2–14].The antibodies were used as 50% ammonium sulfate fractions of ascites. Sera from patients with asthma or atopic dermatitis were selected as a source of IgE Ab. Collection of sera used in this study was approved by the Human Investigation Committee of the University of Virginia. Patients were skin-test positive to *D. pteronyssinus* (>4×4 mm weal on skin-prick testing) and had serum IgE Ab to Der p 2 (measured by MoAb modified RAST as previously described) [12].

Competitive inhibition assay

The relative ability of variant antigen to interact with MoAb and IgE Ab was determined by competitive inhibition in a modified ELISA assay as follows: antigen was mixed with Ab to give a final antigen concentration of 0.01–100 μg/mL. The Ab concentration was predetermined to give an end-point OD_{405} of 1 in the ELISA in the absence of any inhibitor. All antigen and Ab solutions were prepared in phosphate buffered saline with 0.05%Tween-20, 1%BSA. The antigen-Ab mixes were allowed to equilibrate overnight at 4 °C and then were pipetted in duplicate into wells of a plastic microtitre plate that had been coated with rDer p 2 at 20 μg/mL. The plates were incubated at room temperature for 2 h and then processed as a standard ELISA using peroxidase conjugated goat anti-mouse IgG or goat anti-human IgE as the secondary antibody. The positive control was rDer p 2 as inhibitor and the negative controls were GST and saline-BSA diluent. Results were expressed as the percentage inhibition of the reaction of Ab in the absence of any inhibitor.

Results and discussion

Structural predictions

Epitope mapping by site-directed mutagenesis is most often applied to proteins of known three dimensional structure, where specific surface residues can be targeted [6–9]. The tertiary structure of Der p 2 has not yet been determined and previous studies in our laboratory suggested that the intact structure, stabilized by three disulfide bonds, was essential for antibody binding [19,20]. In addition, unlike other *Dermatophagoides* allergens, Der p 2 shows no sequence homology with proteins of known three- dimensional structure, so that a structure can not be modelled on other protein structures. This makes the use of predictive algorithms particularly useful for investigating the antigenic structure of Der p 2. Figure 1 shows the secondary structure profiles of the Der p 2 primary amino acid sequence of 129 residues. The three algorithms (Neural net, Chou-Fasman, Garnier-Robson) showed similar profiles. Regions of α-helix were predicted at the amino and carboxy termini and were flanked by regions predicted to be β-sheet, at residues 10–20 and 100–110. The region of sequence between residues 30 and 60 showed overlapping helix and sheet profiles. Turns were predicted by the Garnier-Robson method between residues 20–30 and 70–80, coincident with the C21-C27 and C73-C78 disulfide bonds.

Following the assumption that antigenic determinants are composed of amino acid residues on the protein surface, predictive algorithms were used to target potential surface residues on Der p 2 that scored highest for hydrophilicity and flexibility. These profiles are also shown in Fig. 1. The primary sequence was subjected to the hydrophilicity scoring system of Hopp and Woods, and the scores were averaged over a window of seven residues [22]. Peaks of hydrophilic sequence occurred at the amino-terminus, between residues 20–30, 40–50, 80–90, and additional peaks were centered around the lysine residue at position 100 and aspartic acid residues at positions 113 and 114. Huang *et al.* studied the correlation between hydrophilicity and solvent accessible surface and between chain flexibility and mobility for proteins of known structure. These investigators found that the hydrophilicity profile correlated more consistently with surface area while flexibility had only a limited correlation with mobility [21]. The flexibility profile of Der p 2, generated using the algorithm of Karplus and Schultz, mirrored the hydrophilicity plot; peaks occured between residues 40–50 and at residue 100 (Fig. 1) [23].

Generation of Der p 2 variants

Variants were produced at the two regions that gave the highest hydrophilic and flexible scores: N44, Q45, and N46 were mutagenized as a block and K100 was targeted for point mutagenesis. Oligonucleotides were designed to give all 19 amino acid substitutions at each position with a minimized possibility of introducing stop codons. Variants are shown in Table 1: a total of 22 templates from

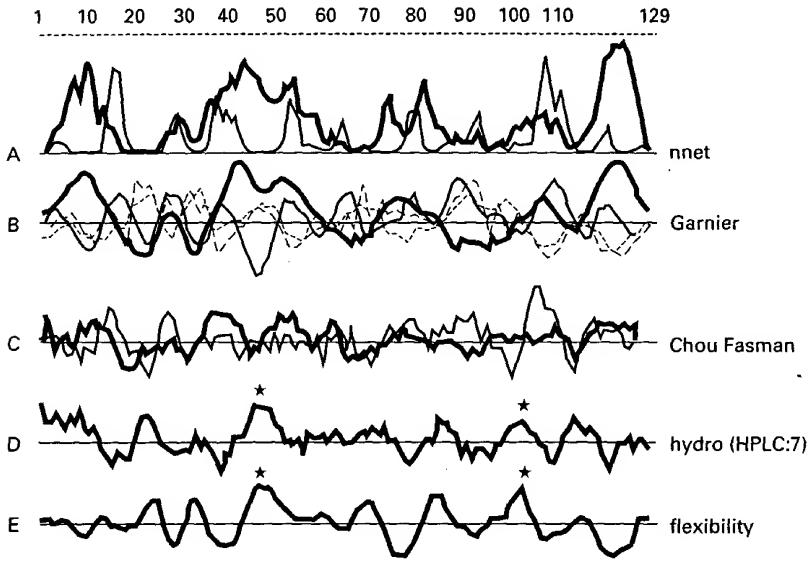

Fig. 1. Analysis of the secondary structure, hydrophilicity, and flexibility of Der p 2. The primary amino acid sequence of Der p 2 (amino acids 1–129) is represented by the dashed line. This sequence was subjected to secondary structure predictions using the algorithms of Holley and Karplus, 'Neural net' (A), Garnier-Robson (B), Chou Fasman (C). Alpha-helical regions are shown by the heavy line and beta sheet regions are shown by the light line. The Garnier-Robson algorithm (B) also predicts turns (long dashes) and random structure (short dashes). The hydrophilicity profile using the Hopp and Woods algorithm is shown in (D) and the flexibility profile using the algorithm of Karplus and Schultz is shown in (E). The residues targeted for mutagenesis are designated (★).

the N44-Q45-N46 targeted mutagenesis reaction were sequenced. Four wild-type sequences were recovered, 10 variants with changes at all three positions, and six variants with one, two, or three codon changes. One variant was sequenced that contained a stop codon at amino acid 46. From the mutagenesis reaction targeted to lysine 100, 10 templates were sequenced: one wild-type sequence was recovered, six point mutants, one stop codon, and two insertions of a single amino acid between lysine 100 and serine 101. The efficiency of mutagenesis was greater than 80%.

Table 1. rDer p 2 variants generated by site-directed mutagenesis

Asn44-Gln45-Asn46 variants:	
Thr Pro Pro	His Pro Leu
Tyr Pro Phe	Ser Leu Leu
His His Leu	Asn Pro His
Tyr His Ile	Asn Leu Pro
Ile His His	Asn His Phe
Pro Thr Tyr	Tyr Gln Pro
Thr Pro Stop	Thr Ser Asn
Gln Thr Thr*	Ser Lys Asn
Pro Pro His*	
Lys100 Variants:	
Asp	
Pro	
Stop	
Arg*	
Thr*	

* Variants selected for Ab binding studies.

Localization of antigenic determinants

Two variants at N44-Q45-N46 were evaluated and the results of the competitive inhibition assays are summarized

Table 2. ELISA inhibition profiles of rDer p 2 and variants[a]

	% Inhibition of antibody binding						
	Monoclonal antibodies				IgE antibodies		
Inhibitor	αDpX	15E11	7A1	13A4	pool[b]	L.W.	F.D.
rDer p 2	80	80	85	85	90	85	80
Surface variants:							
N44Q	75	40	85	85	90	82	88
N44P	9	< 2[c]	50	8	40	30	33
K100T	80	55	30	5	90	82	82
K100R	80	60	75	2	90	83	87
Cysteine variants:[c]							
C21S	80	35	80	75	80	72	93
C73R	14	< 2	65	13	17	53	23
C119Y	80	45	85	80	85	90	92

[a]Maximal inhibition of Ab binding in ELISA using 100 μg/mL rDer p 2 or variant.
[b]Serum pool from seven house dust mite allergic patients.
[c]Values less than the negative control are recorded as < 2; GST and PBS-1%BSA were used as negative controls and showed a maximum of 2% inhibition.
[d]Substitutions at cysteine residues lack the corresponding disulfide bond.

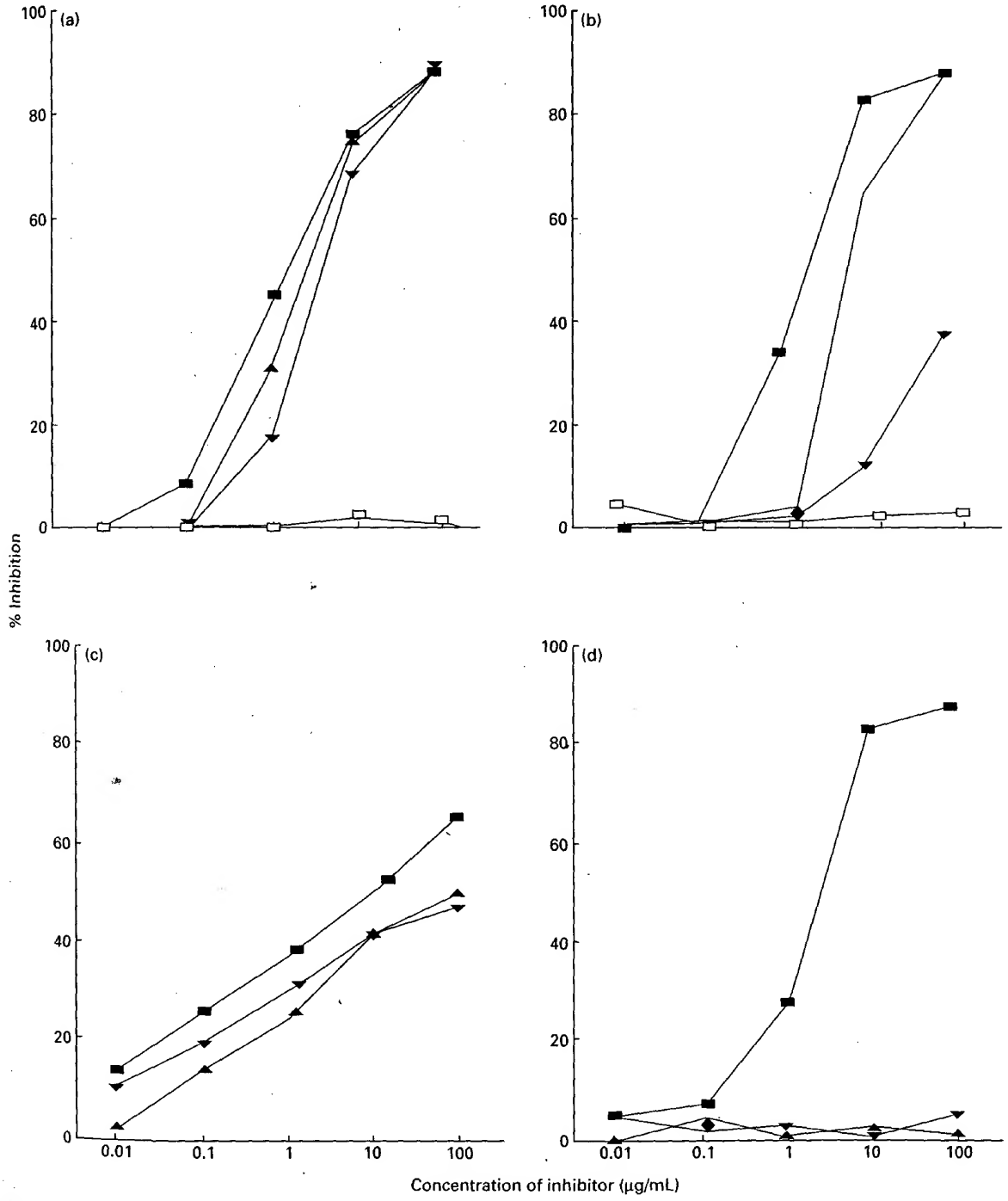

Fig. 2. Monoclonal antibody binding to rDer p 2 variants at K100. Increasing concentrations of rDer p 2 or rDer p 2 variants were used to block binding of MoAb αDpX (a), MoAb 7A1 (b), MoAb 15Ell (c) and MoAb 13A4 (d) to rDer p 2 in a modified ELISA. Inhibitor antigens were rDer p 2 (■), K100R (▲), K100T (▼), and GST (□), the negative control. The results are presented as the percentage inhibition of MoAb binding in the absence of inhibitor.

in Table 2. For this analysis, we considered maximum inhibition of Ab binding of ≤ 50% as a significant reduction in antigenicity. Conservative substitution N44Q gave comparable inhibition curves to rDer p 2 for MoAb αDpX, 7A1, 13A4, and for IgE Ab from four individual patients and a serum pool from seven additional patients. However, this variant had reduced antigenicity for MoAb 15E11, inhibiting binding up to 40% at the highest concentration tested. This result suggests that the 44–46 region is important to the epitope defined by MoAb 15E11. By contrast, the N44P substitution, predicted to introduce a 'helix breaker' motif, failed to inhibit binding of the four MoAb as well as IgE Ab, suggesting that this substitution has effects on several epitopes. Less than 20% inhibition of binding was detected for MoAb αDpX, 15E11, and 13A4 and MoAb 7A1 was inhibited by 50%. IgE Ab binding to the N44P variant was significantly reduced; N44P showed only 30–40% inhibition of binding at 100 μg/mL. Table 2 shows data for two patients, L.W. (atopic dermatitis) and F.D. (asthma). All four individuals and the serum pool gave unique inhibition curves, however, the pattern was consistent; that is, the N44P variant showed markedly decreased antigenicity. This dramatic reduction in reactivity with the polyclonal IgE Ab was also seen in a panel of Der p 2 variants lacking one of the three disulfide bonds (Table 2). We have previously shown that these variants are 10–100-fold less reactive in skin testing and propose that such modified allergens would provide alternative reagents for immunotherapy, with reduced risk of side-effects [20]. The N44P variant provides an additional example of a strategy for modification of IgE binding determinants.

Table 2 also summarized the binding assays using variants at K100. The binding inhibition dose response curves using these substitutions are shown in Fig. 2. The K100R substitution is conservative, maintaining the positively charged side chain, while the K100T substitution removes this charge and introduces a less hydrophilic side chain. These variants gave overlapping inhibition curves with rDer p 2 for MoAb αDpX and IgE Ab. The other MoAb showed distinct binding patterns. The MoAb 13A4 was inhibited by < 10% using either substitution, suggesting that K100 is critical to the epitope structure. Inhibition of binding of MoAb 15E11 reached 55% and 60% with K100T and K100R, respectively, at the maximum concentration tested, indicating that this amino acid may contribute to the 15E11 epitope. However, these values did not meet the criteria set for significant loss of antigenicity. Inhibition of binding of MoAb 7A1 was slightly reduced for K100R but reached only 30% for K100T, thus the positive charge of the side chain at position 100 is critical for MoAb 7A1 binding (Table 2). These results strongly suggest that K100 forms an important part of the epitope defined by MoAb 13A4 and 7A1, two MoAb previously shown to bind to the same region of Der p 2 by cross-inhibition studies [13]. The differential effects of the two substitutions on binding of each MoAb suggest differences in the fine specificity of these antibodies, and underscores the importance of testing multiple substitutions at a given position.

The MoAb 15E11 was sensitive to substitutions at both N44 and K100, although the level of inhibition by the K100 variants did not reach the cut off of ≤ 50% inhibition of binding. It may be that residues 44–46 and 100 are juxtaposed on the surface of Der p 2 and fall within the 15E11 epitope. Recently, we have been able to obtain preliminary NMR spectra of Der p 2 using ^{15}N and ^{13}C labelled recombinant allergen (G. Mueller *et al.* manuscript in preparation). Once the structure is known, we will be able to refine and expand this analysis to residues surrounding N44 and K100 to provide a more detailed map of the antigenic surface of Der p 2.

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